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Research Article

Promising Antibacterial Effects of Silver Nanoparticle-Loaded Tea Tree Oil Nanoemulsion: a Synergistic Combination Against Resistance Threat

Roqya Najafi-taher,[1] Behnaz Ghaemi,[1] Sharmin Kharazi,[1] Samira Rasoulikoohi,[1,2] and Amir Amani[1,3,4]

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Abstract. Highly resistant pathogens may be developed in patients with immune disorders after prolonged exposure to antibiotics, a growing threat worldwide. In order to overcome these problems, this study introduces a new class of engineered nanosystems comprising of tea tree oil nanoemulsion (TTO NE) loaded with Ag nanoparticles (NPs). Silver shows a strong toxicity towards a wide range of microorganisms. Also, TTO NE could be employed as a promising and safe antimicrobial agent for local therapies of bacterial infections. The nanosystem was prepared by low-energy method. Mean droplet size of the NE was found to be 17.7 nm. Results of the antibacterial assays showed promising ability of the designed nanosystem for eradication of Gram-positive and Gram-negative bacteria (95%). Also, it was shown that introducing colloidal Ag NPs to the TTO NE exerted a synergistic effect against *Escherichia coli* (FIC 0.48) while only an additive effect was observed against *Staphylococcus aureus* (FIC 0.75). The antibacterial effects of TTO NE+Ag NPs together with their compatibility with human cells can present them as a suitable candidate to fight against the antibacterial resistance threat.

KEY WORDS: tea tree oil nanoemulsion; resistant bacteria; silver nanoparticle; synergism.

INTRODUCTION

Microbial infections are exceptions in our body harmonics which cause major health problems throughout the world (1). Although antibiotics has been successfully employed as "miraculous drugs" to treat lethal infectious diseases for a long time, inappropriate and disproportionate use of them has resulted in a rapid rise in the incidence of drug-resistant microorganisms (2). Followed by antibiotic resistance, new bacterial strains appear which are simultaneously resistant against numerous antibiotics (3). So, developing an effective and biocompatible antimicrobial agent with no or minimum microbial resistance is a vital issue in this field.

During past years, application of nanomaterials in different aspects of medical sciences has increased. One of which is employing nanoparticles as an efficient alternative antibiotic agent. Silver nanoparticles (Ag NPs) are a common type of antimicrobial agents that show a strong cytotoxicity against a wide range of microorganisms. Antimicrobial activity of Ag NPs is related to generation of free radicals (4). Additionally, Ag NPs bind to DNA (5) and block transcription or attach to cell surface components and interrupt bacterial respiration/adenosine triphosphate (ATP) synthesis (6). Cationic silver also binds to negatively charged components of proteins and nucleic acids (7), thereby causing structural changes and deformations in bacterial cell walls, membranes (8), and nucleic acids (9). Furthermore, silver ions are generally well known to interact with a number of electron donor functional groups such as thiols, phosphates, hydroxyls, imidazoles, indoles, and amines (10).

Among other nanostructures, nanoemulsions offer substantial advantages including ability to be filtered, relatively low viscosity, and improvement of biological and physicochemical properties as well as ease of production and scale-up. Tea tree oil nanoemulsions (TTO NEs) may be employed as promising antimicrobial agents for treatment of bacterial and fungal pneumonia and acne (11). TTO, which is obtained by steam distillation of *Melaleuca alternifolia* leaves, is a natural essential oil with strong antimicrobial efficacy and little drug resistance (12). Antibacterial activity of TTO NE includes influencing potassium homeostasis (13), glucose-dependent respiration (14), and whole cell lysis and transcriptional alteration (15).

In a previous report, concurrent use of free-silver ion and bulk TTO has shown proper antimicrobial activity against

Roqya Najafi-taher and Behnaz Ghaemi contributed equally to this work.

[1] Department of Medical Nanotechnology, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran.
[2] Antimicrobial Resistance Research Center, Rasoul-e-Akram Hospital, Iran University of Medical Sciences, Tehran, Iran.
[3] Medical Biomaterials Research Center (MBRC), Tehran University of Medical Sciences, Tehran, Iran.
[4] To whom correspondence should be addressed. (e-mail: aamani@tums.ac.ir)

Pseudomonas aeruginosa, *Staphylococcus aureus*, and *Candida albicans* (16). A similar finding was also reported by use of liposome-loaded silver ion and bulk TTO (17). Considering the fact that a combination of TTO NE with Ag NPs may play an important role in control of resistant bacteria, in the present study, we focused on producing a TTO NE containing Ag NPs in its aqueous phase. Subsequent to cytotoxic investigations, antibacterial activity of the nanosystem was determined against clindamycin-resistant *Escherichia coli* (ATCC25922) and *S. aureus* (ATCC25923). Synergistic antibacterial effect of TTO NE and Ag NPs was also evaluated.

MATERIALS AND METHODS

Materials

Tea tree oil, Tween 80, Span 80, ethanol, silver nitrate (purity > 99%), sodium citrate, and sodium borohydride were purchased from Sigma-Aldrich (USA).

Microorganism

Resistant bacterial strains *E. coli* (ATCC25922) and *S. aureus* (ATCC25923**)** were supplied from the Antimicrobial Resistance Research Center (Rasoul-e-Akram hospital, Tehran, Iran), then were cultured in Nutrient Broth and kept at 37°C overnight to prepare 0.5 McFarland standard suspension (18).

Preparation of Ag NPs

Ag NPs were prepared using sodium borohydride as reducing agent and sodium citrate as stabilizer. Briefly, Ag NPs were synthesized by adding 0.5 ml $AgNO_3$ 0.01 M into 20 ml stirring solution of sodium citrate 1 mM. After 10 min, 0.5 ml sodium borohidrate 0.01 mM was added to this solution and the obtained mixture was stirred for 10 min. NPs were synthesized at room temperature (19).

Preparation of Ag NP-Loaded TTO NE (TTO NE+Ag NPs)

A mixture of Tween 80 and Span 80 (72:28) was selected as a surfactant. In order to prepare a clear nanoemulsion, aqueous phase was prepared by dissolving the surfactant mixture and ethanol in water under magnetic stirring. Then, the aqueous phase was mixed with the oil using a low-shear mixer for 15 min to obtain a transparent emulsion. Subsequently, Ag NPs were added to the system to obtain a TTO NE containing Ag NPs as shown schematically in Fig. 1(a).

Characterization of Prepared Nanoemulsion

UV-Vis Absorption Spectroscopy

UV-Vis absorption spectra of samples were carried out by a Cary100 Conc (Varian, Australia) with an optical resolution of 0.01-nm full width at half maximum (FWHM). Spectrum responses were recorded from 200 to 800 nm with 1-nm steps in a 4 × 1 × 1 cm path quartz cuvette.

Transmission Electron Microscopy

Size and morphology of the prepared nanoemulsion were evaluated by transmission electron microscopy (TEM). Samples were prepared by dropping the 1 mM concentration nanoemulsion onto the lacey carbon film of a 200-mesh Cu grid, after solvent evaporation at ambient temperature.

Dynamic Light Scattering and Zeta Potential Analysis

Particle size, distribution, and zeta potential of TTO NE were determined using dynamic light scattering (DLS) (Brookhaven Instruments, USA). Nanoemulsions were diluted (1:10) with water prior to the experiment.

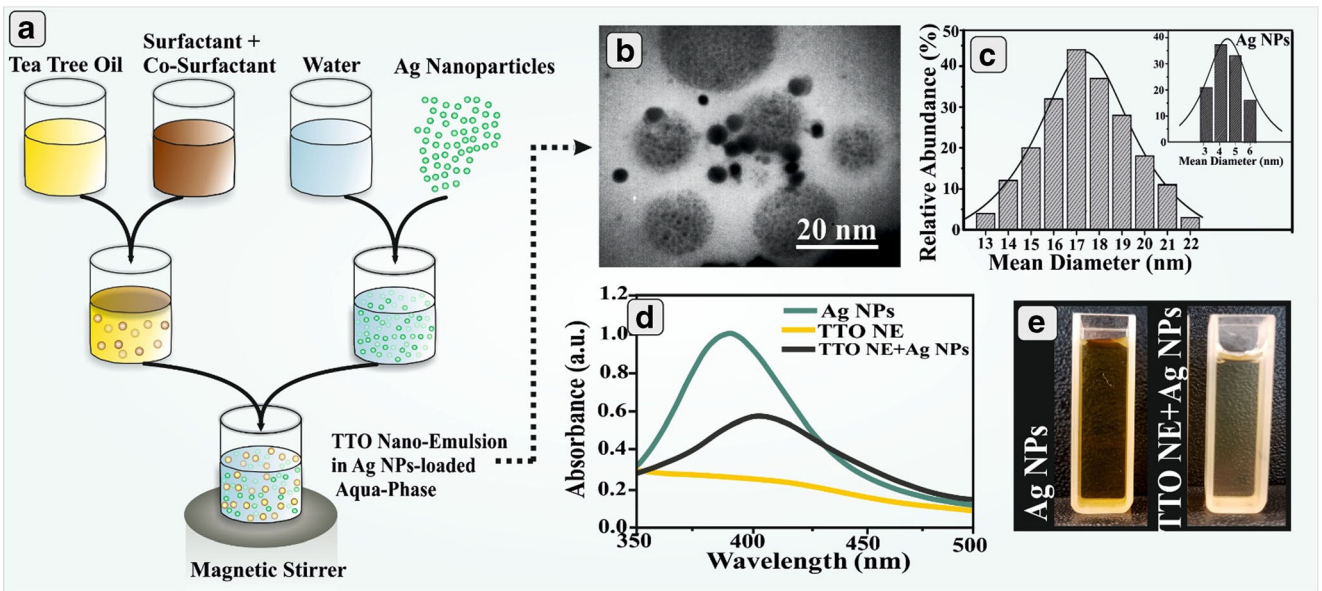

Fig. 1. Schematic illustration of preparation procedure of TTO NE+Ag NPs (**a**), TEM micrograph and size distribution of TTO NE and Ag NPs as inset (**b**, **c**). UV-Vis spectroscopy of TTO NE+Ag NPs and Ag NPs, as well as TTO (**d**). Optical images of Ag NPs and TTO NE+Ag NPs (**e**)

Toxicity Assessment on Human Cells

Cell Culture

Human lung fibroblast cells line (MRC-5) were assessed *in vitro*. Cells were purchased from National Cell Bank of Pasteur Institute (Iran) and cultured at a density of 1×10^4 cells per well (96-well plate) and cultivated in growth medium of Dulbecco's Modified Eagle Medium (DMEM F12, Gibco Invitrogen, UK) and RPMI 1640 (Merck, Germany). They were supplemented with 10% fetal bovine serum (Sigma, USA) and 1% penicillin-streptomycin-glutamine (Gibco Invitrogen, UK). Cells were stored in a humidified incubator at 37°C and 5% CO_2 atmosphere.

Cell Treatments

Six samples as detailed in Table I were added to the cell media after 24 h of culture and incubated overnight. Samples studied included Ag NPs, TTO NE, TTO NE containing Ag NPs (TTO NE+Ag NPs), TTO emulsion, micellar solution (*i.e.*, all the NE ingredients except TTO), bulk TTO, and PBS as control negative. Treated cells were subsequently assessed to analyze the toxicity effects of the samples.

MTT Assay

Apoptosis induction of the samples was determined by MTT assay where 20 µl of MTT (5 mg/ml, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma, Germany) was added to each well and incubated for 4 h at 37°C. The color absorbance at 590 nm was then quantified using an ELISA Reader (Biotek, ELX808) and presented as percentage of controls.

Antibacterial Efficiency Studies

Disc Diffusion Test

Blank discs were bought from Patan Teb (Iran) and impregnated with samples. Discs were then applied to Muller agar growth medium after culturing *S. aureus* and *E. coli*. Then, zone diameters were measured after a 24-h incubation. Antimicrobial tests were conducted under sterile condition.

Bacterial Viability Assay

In order to evaluate the viability of resistant *S. aureus* and *E. coli* bacteria after a 24-h treatment with samples listed in Table I, LIVE/DEAD® BacLight Bacterial Viability Kit (Invitrogen, USA) was employed. The kit consists of two dyes, SYTO®9 and propidium iodide, that penetrate bacterial cells and show bacterial cell viability as a function of membrane integrity. SYTO®9, a green fluorescent at 530 nm upon excitation at 488 nm, is capable of passing the intact membrane of both viable and dead bacterial cells. Propidium iodide (PI, red fluorescence at 620 nm upon excitation at 488 nm), a nucleic acid stain, enters compromised membranes of bacterial cells that are either dead or dying. The intensity of fluorescence was measured using a 96-well fluorescence microplate reader for each sample.

Minimum Inhibitory Concentration

Minimum inhibitory concentration (MIC) of the samples was determined by the twofold microdilution method with sterile flat-bottom 96-well polystyrene plates. The test samples were serially diluted twofold with nutrient soup. The culture-inoculated plates were incubated at 37°C for 48 h, and the growth was recorded spectrophotometrically at 600 nm. The MIC values were evaluated as specific concentrations where a noticeable reduction in color formation due to bacterial growth inhibition was noted. All experiments were carried out in triplicates (20).

Assessment of Synergistic Effects

Synergistic effects of Ag NPs in combination with TTO NE were assessed using the microdilution technique. The fractional inhibitory concentration was calculated for two antimicrobial agents as

$$\mathrm{FIC(AgNPs)} = \frac{\mathrm{MIC\,(TTO\,NE + AgNPs)}}{\mathrm{MIC\,(AgNPs)}}$$

$$\mathrm{FIC\,(TTO\,NE)} = \frac{\mathrm{MIC\,(TTO\,NE + AgNPs)}}{\mathrm{MIC\,(TTO\,NE)}}$$

$$\sum \mathrm{FIC} = \mathrm{FIC\,(AgNPs)} + \mathrm{FIC\,(TTO}\,\textit{nanoemulsion})$$

where $\sum \mathrm{FIC} \leq 0.5$ is a sign of synergism, > 0.5–1 of addition, > 1 of indifference, and ≥ 2 of antagonism (21).

Statistical Analysis

All experiments were performed in triplicate. The cell viability assessment was analyzed by SPSS 19.0 statistical

Table I. Details of Samples Studied for Cytotoxicity and Antibacterial Assays

Formulation	TTO (%)	Surfactant (Tween 80: Span 80) (72:28) (%)	Co-surfactant (ethanol) (%)	Water (%)	Ag NPs (25 ppm) (%)
Ag nanoparticles	–	–	–	–	100
TTO NE+Ag NPs	5	19	19	–	57
TTO NE	5	19	19	57	–
TTO emulsion	5	5	5	85	–
Bulk TTO	100	–	–	–	–
Micelle	–	19	19	62	–

package (SPSS Inc., USA) and in case of normally distributed data, a two-tailed Student's *t* test was performed. *P* values of less than 0.001, 0.001 to 0.01, and 0.01 to 0.05 were considered as significant.

RESULTS AND DISCUSSION

Morphological Analyses and Size Distribution of TTO NE+Ag NPs

Figure 1(a) schematically represents the major steps involved in producing TTO NE+Ag NPs. Obtained nanoparticles were evaluated for morphology, size, and size distributions with TEM and UV-Vis absorption spectroscopy as well as DLS. TEM results demonstrate an average size of 17.5 and 4.5 nm with spherical morphologies for TTO NE and Ag NPs, respectively (Fig. 1(b, c)). UV-Vis absorption spectra in the region of 200–800 nm displayed an absorption peak at 390 nm for Ag NPs and a 400-nm shifted peak for TTO NE+Ag NPs. This is attributed to the distinctive surface plasmon resonance peak of silver nanoparticles (Fig. 1(d)) (22,23). Peak displacement in UV-Vis spectra of Ag-loaded TTO NE was due to environmental changes (24), even though TTO NE does not have any important absorption in this region.

Droplet size distribution, polydispersity index (PDI), and zeta potential are very important parameters when it comes to characterization of nanoemulsion formulations. PDI is related to the uniformity of droplets within the nanoemulsion system and zeta potential is concerned with the stability of the systems. Results of DLS measurement showed a mean size and PDI of 17.5 and 0.162 nm for TTO NE and 17.7 and 0.221 nm for TTO NE+Ag NPs. Apparently, the presence of the small Ag NPs increases PDI of TTO NE+Ag NPs in comparison with TTO NE. The small obtained PDI values indicate uniformity in the nanoemulsion, which is well consistent with the TEM micrograph. Also, zeta potential measurement demonstrated that loading Ag NPs in TTO NE causes zeta potential changes from -17.75 ± 0.46 to -29.24 ± 1.28 mV which possibly improves the stability of TTO NE. Previous reports demonstrate that the magnitude of zeta potential gives an indication of the potential electrostatic stability of the NE (25).

Toxicity of TTO NE+Ag NPs in Human Cells

Along with antimicrobial properties, components of TTO, which can penetrate the skin in topical delivery or increase penetration of other components, may lead to toxicity. In order to evaluate toxicity effects of the prepared nanoemulsion on cells, human lung fibroblast (MRC-5) was treated with various components of NE as well as different Ag concentrations of TTO NE+Ag NPs (Fig. 2). Figure 2a shows the toxicity of the samples under study. In comparison with phosphate buffer saline (PBS) as negative control, Ag NPs as effective components of antibacterial nanoemulsion showed significant toxicity (25%) at 14 μg/ml concentration. A previous research compared the toxicity of Ag NPs with nanoparticles of molybdenum (MoO_3; 30, 150 nm), aluminum (Al; 30, 103 nm), iron oxide (Fe_3O_4; 30, 47 nm), and titanium dioxide (TiO_2; 40 nm). Results showed that mitochondrial function decreased significantly in cells exposed to Ag NPs at 20–50 μg/ml in comparison with the other NPs (26). Furthermore, our results did not show any significant toxicity for TTO NE, TTO NE+Ag NPs, TTO emulsion (5 mg/ml), and micelles (19 mg/ml surfactants [Tween 80 and Span 80 (72:28)] and ethanol) as illustrated in Fig. 2a. The highest toxicity was recorded for bulk TTO (as pure oil) which caused 75% cell death. Previous research introduced 12.5 nm TTO NEs as promising local therapies of fungal and bacterial pneumonia without any significant human cell toxicity and

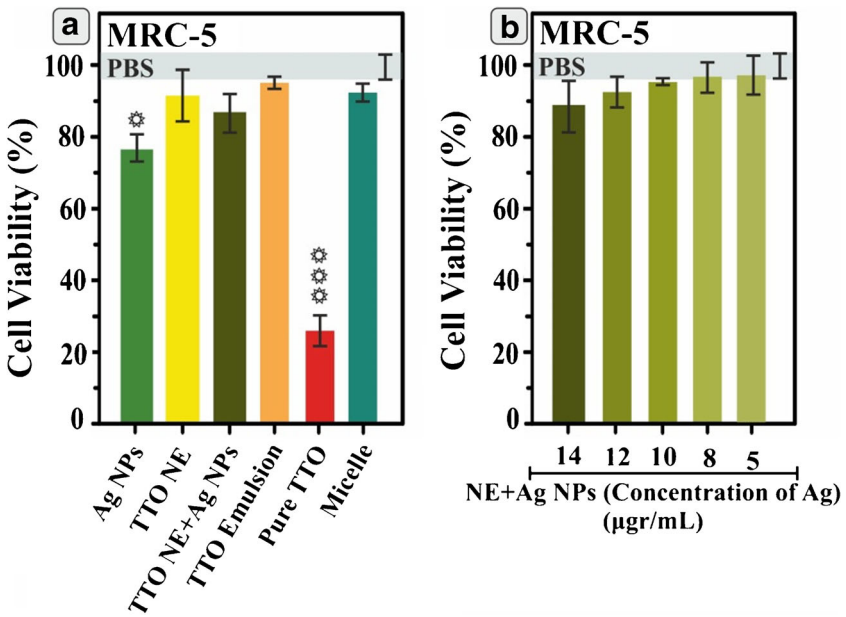

Fig. 2. Survival of MRC-5 after treatment with Ag NPs (14 μgr/ml), TTO NE, TTO NE+ 14 μgr/ml Ag NPs, TTO emulsion, micelles (surfactants, ethanol, and water) and pure bulk of TTO (**a**). Cell viability of MRC-5 cell line after incubation with different concentrations of Ag NPs in TTO NE (**b**)

in vivo adverse events (27). Another report showed that 5% TTO can be safely applied to the skin without any skin irritation (28).

Furthermore, toxicity of TTO NE+Ag NPs with different concentrations of Ag NPs were evaluated on MRC-5 cells (Fig. 2b). A concentration-dependent dampening of toxicity was observed when concentration of Ag NPs decreased to 5 μg/ml. Previous researches demonstrated a concentration-dependent toxicity of Ag NPs both *in vitro* and *in vivo* (29,30). In total, the results do not show a considerable cytotoxicity profile when Ag NPs are loaded in TTO NE up to 12 μg/ml. We demonstrate that TTO NE+Ag NPs did not show any significant toxicity on human cells.

Antimicrobial Activity

Nanoemulsions have been reported as innovative and promising agents against antibacterial threat (31). Due to differences between the bacteria's cell wall and plasma membrane, we evaluated lab strains of *E. coli* (Gram negative) and *S. aureus* (Gram positive). LIVE/DEAD assay, disc diffusion, and microdilution methods were conducted on the standard and tetracycline-resistant *S. aureus* and *E. coli* after 24 h of growth, in the presence of samples under study as well as an antibacterial drug (tetracycline) (Fig. 3).

Obtained results of LIVE/DEAD bacteria assay showed that, in the presence of TTO NE+Ag NPs, the percentage of dead resistant bacteria was significantly higher (~ 85%) than that in other samples. Remarkably, the TTO NE+Ag NPs exhibited significant differences in the percentage of dead bacteria in comparison with the controls (*i.e.*, PBS and tetracycline) for both resistant bacteria types. TTO showed also considerable antibacterial effects. However, its toxic effect against human healthy cells limits its potential use as an antibacterial agent, having mentioned that the concentration of TTO is 20 times more than that of TTO NE.

Also, bacterial disc diffusion assays were used to determine the antimicrobial activity of the samples. The diameters of inhibition zones around the discs are shown in Fig. 3b. Results showed that antibacterial activities of Ag NPs in the presence of TTO NE increased against the resistant bacteria as well as standard strains. Interestingly, the antibacterial effects of TTO NE+Ag NPs on resistant strains were similar to standard ones, whereas the antibacterial effects of tetracycline against resistant bacteria were much less. Similar to that above, TTO showed important antibacterial activity.

The antibacterial activity of silver, silver ions, and silver compounds has been thoroughly investigated (32). A survey of recent literature showed remarkable findings on the bactericidal activity of silver nanoparticles (Ag NPs) against *E. coli* and multidrug-resistant *S. aureus* (33). Another research reported that the antibacterial activity of Ag NPs against *E. coli* depends on the shape of the nanoparticles (34).

Furthermore, because of its antifungal, anti-inflammatory, and antiviral activities as well as low toxicity profiles, TTO is a promising source for new phytotherapeutic agents for treatment of bacterial-resistant threat (35). Literature describes the remarkable performance of TTO microemulsion, sunflower oil microemulsion (36), and eucalyptus oil NE (37) as antimicrobial agents. Also, TTO has been reported as burn dressing (38), oral hygiene formulation (39), fungicidal emulsion (40), and topical medicine for treatment of viral lesions (41). Researches showed that the antibacterial and antifungal properties of TTO components are due to the physicochemical properties of the oil which is comprised of hydrophilic compounds with sufficient lipophilicity which allow the oil to partition preferentially into biological membranes. This causes bilayer expansion, inhibition of respiration, loss of membrane integrity, and inhibition of embedded membrane enzymes (42). Previous researches showed membrane disruption and related leakage of potassium ions from TTO-treated *E. coli*, *S. aureus*, and *Candida albicans* (43). Carson *et al.* assessed the release of nucleic

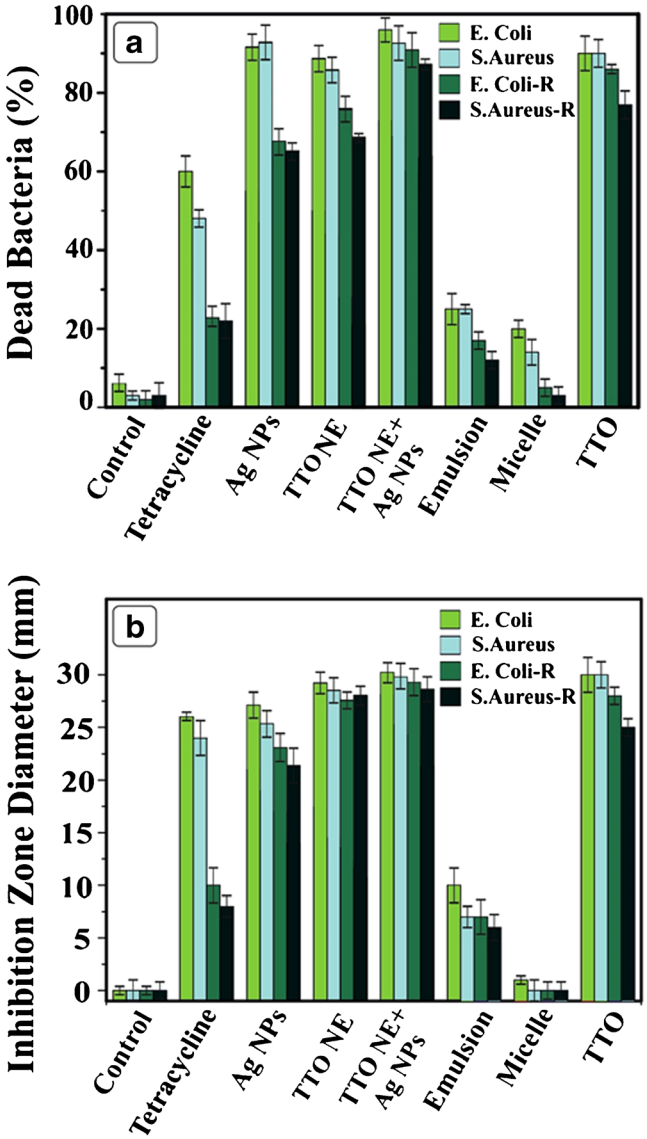

Fig. 3. Percentage of dead bacteria treated by TTO NE+Ag NPs (14 ppm Ag NPs), Ag NPs (14 ppm), and bulk TTO, as well as other samples, in 24 h, against standard and resistant strains (R) of *E. coli* and *S. aureus*. Error bars represent standard deviation for ten independent experiments. Experiments with tetracycline were carried out on bacteria for confirmation of strong antibacterial effects of prepared NE (**a**). Inhibition zone of growth bacteria in disc diffusion test with different components of TTO NE+Ag NP (**b**) controls are untreated samples in both figures

acids from *S. aureus* after treatment with TTO and the results showed extensive damage to the cell membranes (15). Additionally, nanoemulsions have shown promising antimicrobial activities compared with corresponding emulsion formulations (44,45). So, TTO nanoemulsion loaded with Ag NPs may also be an effective antimicrobial agent specially for topical applications (46).

In terms of mechanism of antibacterial effects of Ag nanoparticles, two different mechanisms have been reported:

- Bactericidal effects of silver nanoparticles due to release of Ag^+ ions which disrupts membrane structure of bacteria. It has been proven that Ag^+ ions trigger mitochondrial-dependent cytotoxic pathway in the human cells.
- Reactive oxygen species (ROS) are generated by transfer of free electrons on the surface of metallic nanoparticles to the environmental biomolecules. ROS generation is the principal antibacterial pathway at low concentration of silver nanoparticles (47), while human cells could tolerate the excess amount of oxidative stress due to their high antioxidant capacity (48).

In order to optimize the amount of Ag NPs which is harmful to human cells, the antibacterial effects of TTO NE+Ag NPs and standard and tetracycline-resistant lab strains of *E. coli* and *S. aureus* were treated with different concentrations of Ag NPs in NEs (Fig. 4). TTO NE+Ag NPs showed significant antibacterial effect (*i.e.*, 95% for standard and 90% for tetracycline-resistant bacteria) at the highest concentration applied (14 μg/ml). Decrease of Ag NP concentration in TTO NE+Ag NPs to 8 ppm has not shown any significant effects on death of *E. coli*. However, the amount of dead *S. aureus* bacteria was decreased to 65% when the concentration of Ag NPs decreased to 8 ppm. So, concentration of Ag NPs is an important factor against *S. aureus*. Reports showed that the concentration of Ag NPs plays effective roles in killing Gram-positive bacteria (49). So,

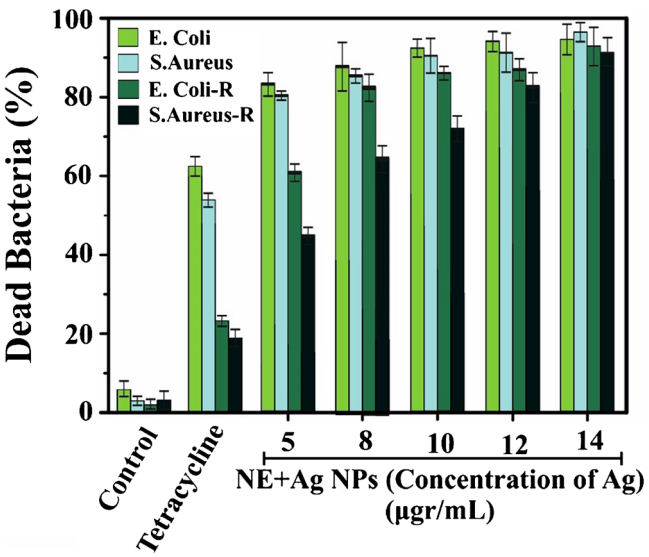

Fig. 4. Concentration dependence of antibacterial effects of Ag NPs in TTO NE against standard and resistant (R) strains of *E. coli* and *S. aureus* in comparison with tetracycline and PBS

Table II. Microdilution Result (MIC) of Ag NPs and TTO NE in Combination and Alone Against Resistant *S. aureus* and *E. coli*

Formulation		R: *E. coli*	R: *S. aureus*
Ag NPs	Alone	25 μg/ml	25 μg/ml
	In combination	8 μg/ml	12.5 μg/ml
TTO NE	Alone	5 mg/ml	5 mg/ml
	In combination	0.8 mg/ml	1.25 mg/ml

it could be concluded that decreasing the amount of Ag NPs in TTO NE+Ag NPs causes a considerable reduction in antibacterial effect against the Gram-negative bacteria (*i.e.*, *E. coli*).

Evaluation of Synergistic Antibacterial Effects for TTO NE and Ag NPs

Due to the antibacterial properties of Ag NPs and TTO NE, we evaluated possible synergistic effects of the materials against resistant Gram-positive and Gram-negative bacteria with the microdilution method. In this way, the minimum inhibitory concentrations (MICs) of TTO NE+Ag NPs on Gram-positive and Gram-negative bacteria were determined. Observed MIC values for Ag NPs were 25 μg/ml for both resistant *E. coli* and *S. aureus*. Also, the TTO NE showed a residual activity against *E. coli* and *S. aureus* with an MIC value of 5 mg/ml (Table II). However, treatment of these microorganisms with TTO NE+Ag NPs showed a considerable increase in antimicrobial activity. The obtained MICs were 8 and 12.5 μg/ml for Ag NPs in combination with TTO NE, against *E. coli* and *S. aureus*, respectively. Also, as shown in Table II, MIC results for TTO NE in combination with Ag NPs were obtained as 0.8 and 1.25 mg/ml against *E. coli* and *S. aureus*, respectively. Table III shows the MIC findings for TTO NE+Ag NPs against both types of Gram-positive and Gram-negative bacteria. Our results demonstrated synergistic effects against clindamycin-resistant *E. coli* and an additional effect for *S. aureus*.

Previous researches also showed similar effect of synergistic and addition for TTO bulk and Ag ion against Gram-negative and Gram-positive bacteria, respectively (16). Also, a synergistic effect for liposome-loaded silver ion and TTO against *E. coli* was shown (17). The difference in effect of TTO NE+Ag NPs against the resistant two strains could be related to compositional difference in cell wall structure of Gram-positive and Gram-negative bacteria (50). In this regard, negatively charged TTO NE+Ag NPs could attack the Gram-negative bacteria by mechanisms like metal depletion as investigated previously (51). Also, cell wall of Gram-

Table III. Antimicrobial Activities of TTO Nanoemulsion and Ag NPs in Combination and Alone Against Resistant *S. aureus* and *E. coli*

Microorganism	FIC (TTO NE)	FIC (Ag NPs)	FIC (NE+Ag NPs)	Inference
R: *E.coli*	0.16	0.32	0.48	Synergism
R: *S. aureus*	0.25	0.5	0.75	Addition

positive bacteria is composed of peptidoglycan with linear polysaccharide chains cross-linked by short peptides which form a three-dimensional rigid structure (52). The rigidity and extended cross-linking provide fewer anchoring site reactions for nanosystems, which work as a barrier.

In the TTO NE, synergistic effects were observed against clindamycin-resistant *E. coli* and additional effects were seen for *S. aureus* in the presence of Ag NPs. Obtained results showed that sufficient antibacterial effects of Ag-loaded TTO NE together with their compatibility with human cells can present a suitable antibacterial agent against resistant threat.

CONCLUSION

There has been growing interest in the usage of broad-spectrum, alternative antimicrobial agents, such as essential oils and metal NPs, to address problems related to increased antibiotic-resistant infections. This report shows that the designed nanosystems of TTO NE+Ag NPs do not cause significant toxicity in human cells. Also, a synergistic antibacterial effect with the combination of Ag NPs and TTO was observed which presents an effective antimicrobial agent for treatment of resistant pathogens.

REFERENCES

1. Martínez JL, Coque TM, Baquero F. Prioritizing risks of antibiotic resistance genes in all metagenomes. Nat Rev Microbiol. 2015;13(6):396. https://doi.org/10.1038/nrmicro3399-c2.
2. Ling LL, Schneider T, Peoples AJ, Spoering AL, Engels I, Conlon BP, et al. A new antibiotic kills pathogens without detectable resistance. Nature. 2015;517(7535):455–9. https://doi.org/10.1038/nature14098.
3. Piddock LJ. Assess drug-resistance phenotypes, not just genotypes. Nat Microbiol. 2016;1(8):16120. https://doi.org/10.1038/nmicrobiol.2016.120.
4. Durán N, Durán M, de Jesus MB, Seabra AB, Fávaro WJ, Nakazato G. Silver nanoparticles: a new view on mechanistic aspects on antimicrobial activity. Nanomedicine. 2016;12(3):789–99. https://doi.org/10.1016/j.nano.2015.11.016.
5. AshaRani P, Low Kah Mun G, Hande MP, Valiyaveettil S. Cytotoxicity and genotoxicity of silver nanoparticles in human cells. ACS Nano. 2008;3(2):279–90.
6. Lim YH, Tiemann KM, Heo GS, Wagers PO, Rezenom YH, Zhang S, et al. Preparation and in vitro antimicrobial activity of silver-bearing degradable polymeric nanoparticles of polyphosphoester-block-poly (L-lactide). ACS Nano. 2015;9(2):1995–2008. https://doi.org/10.1021/nn507046h.
7. Gitipour A, Thiel SW, Scheckel KG, Tolaymat T. Anaerobic toxicity of cationic silver nanoparticles. Sci Total Environ. 2016;557:363–8. https://doi.org/10.1016/j.scitotenv.2016.02.190.
8. Abu-Youssef MA, Soliman SM, Langer V, Gohar YM, Hasanen AA, Makhyoun MA, et al. Synthesis, crystal structure, quantum chemical calculations, DNA interactions, and antimicrobial activity of [Ag (2-amino-3-methylpyridine) 2] NO3 and [Ag (pyridine-2-carboxaldoxime) NO3]. Inorg Chem. 2010;49(21):9788–97. https://doi.org/10.1021/ic100581k.
9. Ramstedt M, Cheng N, Azzaroni O, Mossialos D, Mathieu HJ, Huck WT. Synthesis and characterization of poly (3-sulfopropylmethacrylate) brushes for potential antibacterial applications. Langmuir. 2007;23(6):3314–21. https://doi.org/10.1021/la062670+.
10. Kumar A, Vemula PK, Ajayan PM, John G. Silver-nanoparticle-embedded antimicrobial paints based on vegetable oil. Nat Mater. 2008;7(3):236–41. https://doi.org/10.1038/nmat2099.
11. Lins RF, Lustri WR, Minharro S, Alonso A, de Sousa Neto D. On the formation, physicochemical properties and antibacterial activity of colloidal systems containing tea tree (Melaleuca alternifolia) oil. Colloids Surf A Physicochem Eng Asp. 2016;497:271–9. https://doi.org/10.1016/j.colsurfa.2016.02.024.
12. Yap PSX, Krishnan T, Chan K-G, Lim S. Antibacterial mode of action of Cinnamomum verum bark essential oil, alone and in combination with piperacillin, against a multi-drug-resistant Escherichia coli strain. J Microbiol Biotechnol. 2015;25(8):1299–306. https://doi.org/10.4014/jmb.1407.07054.
13. Flores F, De Lima J, Ribeiro RF, Alves S, Rolim C, Beck RCR, et al. Antifungal activity of nanocapsule suspensions containing tea tree oil on the growth of Trichophyton rubrum. Mycopathologia. 2013;175(3–4):281–6. https://doi.org/10.1007/s11046-013-9622-7.
14. Prabhu S, Poulose EK. Silver nanoparticles: mechanism of antimicrobial action, synthesis, medical applications, and toxicity effects. Int Nano Lett. 2012;2(1):1–10.
15. Carson CF, Mee BJ, Riley TV. Mechanism of action of Melaleuca alternifolia (tea tree) oil on Staphylococcus aureus determined by time-kill, lysis, leakage, and salt tolerance assays and electron microscopy. Antimicrob Agents Chemother. 2002;46(6):1914–20. https://doi.org/10.1128/AAC.46.6.1914-1920.2002.
16. Low W, Martin C, Hill D, Kenward M. Antimicrobial efficacy of silver ions in combination with tea tree oil against Pseudomonas aeruginosa, Staphylococcus aureus and Candida albicans. Int J Antimicrob Agents. 2011;37(2):162–5. https://doi.org/10.1016/j.ijantimicag.2010.10.015.
17. Low W, Martin C, Hill D, Kenward M. Antimicrobial efficacy of liposome-encapsulated silver ions and tea tree oil against Pseudomonas aeruginosa, Staphylococcus aureus and Candida albicans. Lett Appl Microbiol. 2013;57(1):33–9. https://doi.org/10.1111/lam.12082.
18. Andrews JM. Determination of minimum inhibitory concentrations. J Antimicrob Chemother. 2001;48(suppl 1):5–16. https://doi.org/10.1093/jac/48.suppl_1.5.
19. Tavakol S, Hoveizi E, Kharrazi S, Tavakol B, Karimi S, Rezayat Sorkhabadi SM. Organelles and chromatin fragmentation of human umbilical vein endothelial cell influence by the effects of zeta potential and size of silver nanoparticles in different manners. Artif Cells Nanomed Biotechnol. 2017;45(4):817–23. https://doi.org/10.1080/21691401.2016.1178132.
20. The National Committee for Clinical Laboratory Standards (NCCLS) M7-A4. 1997.
21. Yap PSX, Lim SHE, Hu CP, Yiap BC. Combination of essential oils and antibiotics reduce antibiotic resistance in plasmid-conferred multidrug resistant bacteria. Phytomedicine. 2013;20(8):710–3. https://doi.org/10.1016/j.phymed.2013.02.013.
22. Samadi N, Hosseini S, Fazeli A, Fazeli M. Synthesis and antimicrobial effects of silver nanoparticles produced by chemical reduction method. DARU J Pharm Sci. 2010;18(3):168.
23. Kim JS, Kuk E, Yu KN, Kim J-H, Park SJ, Lee HJ, et al. Antimicrobial effects of silver nanoparticles. Nanomedicine. 2007;3(1):95–101. https://doi.org/10.1016/j.nano.2006.12.001.
24. Badawy AME, Luxton TP, Silva RG, Scheckel KG, Suidan MT, Tolaymat TM. Impact of environmental conditions (pH, ionic strength, and electrolyte type) on the surface charge and aggregation of silver nanoparticles suspensions. Environ Sci Technol. 2010;44(4):1260–6. https://doi.org/10.1021/es902240k.
25. Sotomayor-Gerding D, Oomah BD, Acevedo F, Morales E, Bustamante M, Shene C, et al. High carotenoid bioaccessibility through linseed oil nanoemulsions with enhanced physical and oxidative stability. Food Chem. 2016;199:463–70. https://doi.org/10.1016/j.foodchem.2015.12.004.
26. Hussain S, Hess K, Gearhart J, Geiss K, Schlager J. In vitro toxicity of nanoparticles in BRL 3A rat liver cells. Toxicol in Vitro. 2005;19(7):975–83. https://doi.org/10.1016/j.tiv.2005.06.034.
27. Li M, Zhu L, Liu B, Du L, Jia X, Han L, et al. Tea tree oil nanoemulsions for inhalation therapies of bacterial and fungal pneumonia. Colloids Surf B: Biointerfaces. 2016;141:408–16. https://doi.org/10.1016/j.colsurfb.2016.02.017.
28. Malhi HK, Tu J, Riley TV, Kumarasinghe SP, Hammer KA. Tea tree oil gel for mild to moderate acne; a 12 week uncontrolled,

open-label phase II pilot study. Australas J Dermatol. 2017;58(3):205–10. https://doi.org/10.1111/ajd.12465.
29. van der Zande M, Vandebriel RJ, Van Doren E, Kramer E, Herrera Rivera Z, Serrano-Rojero CS, et al. Distribution, elimination, and toxicity of silver nanoparticles and silver ions in rats after 28-day oral exposure. ACS Nano. 2012;6(8):7427–42. https://doi.org/10.1021/nn302649p.
30. Verano-Braga T, Miethling-Graff R, Wojdyla K, Rogowska-Wrzesinska A, Brewer JR, Erdmann H, et al. Insights into the cellular response triggered by silver nanoparticles using quantitative proteomics. ACS Nano. 2014;8(3):2161–75. https://doi.org/10.1021/nn4050744.
31. Lawrence MJ, Rees GD. Microemulsion-based media as novel drug delivery systems. Adv Drug Deliv Rev. 2000;45(1):89–121. https://doi.org/10.1016/S0169-409X(00)00103-4.
32. Warriner R, Burrell R. Infection and the chronic wound: a focus on silver. Adv Skin Wound Care. 2005;18(8):2–12. https://doi.org/10.1097/00129334-200510001-00001.
33. Feng Q, Wu J, Chen G, Cui F, Kim T, Kim JA. Mechanistic study of the antibacterial effect of silver ions on Escherichia coli and Staphylococcus aureus. J Biomed Mater Res. 2000;52(4):662–8. https://doi.org/10.1002/1097-4636(20001215)52:4<662::AID-JBM10>3.0.CO;2-3.
34. Pal S, Tak YK, Song JM. Does the antibacterial activity of silver nanoparticles depend on the shape of the nanoparticle? A study of the gram-negative bacterium Escherichia coli. Appl Environ Microbiol. 2007;73(6):1712–20. https://doi.org/10.1128/AEM.02218-06.
35. Bakkali F, Averbeck S, Averbeck D, Idaomar M. Biological effects of essential oils—a review. Food Chem Toxicol. 2008;46(2):446–75. https://doi.org/10.1016/j.fct.2007.09.106.
36. Anjali C, Dash M, Chandrasekaran N, Mukherjee A. Antibacterial activity of sunflower oil microemulsion. Int J Pharm Pharm Sci. 2010;2:123–8.
37. Saranya S, Chandrasekaran N, Mukherjee A. Antibacterial activity of eucalyptus oil nanoemulsion against Proteus mirabilis. Int J Pharm Pharm Sci. 2012;4(3):668–71.
38. Chin KB, Cordell B. The effect of tea tree oil (Melaleuca alternifolia) on wound healing using a dressing model. J Altern Complement Med. 2013;19(12):942–5. https://doi.org/10.1089/acm.2012.0787.
39. Kothiwale SV, Patwardhan V, Gandhi M, Sohoni R, Kumar AA. Comparative study of antiplaque and antigingivitis effects of herbal mouthrinse containing tea tree oil, clove, and basil with commercially available essential oil mouthrinse. J Indian Soc Periodontol. 2014;18(3):316–20. https://doi.org/10.4103/0972-124X.134568.
40. Shao X, Cheng S, Wang H, Yu D, Mungai C. The possible mechanism of antifungal action of tea tree oil on Botrytis cinerea. J Appl Microbiol. 2013;114(6):1642–9. https://doi.org/10.1111/jam.12193.
41. Gilling D, Kitajima M, Torrey J, Bright KR. Antiviral efficacy and mechanisms of action of oregano essential oil and its primary component carvacrol against murine norovirus. J Appl Microbiol. 2014;116(5):1149–63. https://doi.org/10.1111/jam.12453.
42. Carson C, Hammer K, Riley T. Melaleuca alternifolia (tea tree) oil: a review of antimicrobial and other medicinal properties. Clin Microbiol Rev. 2006;19(1):50–62. https://doi.org/10.1128/CMR.19.1.50-62.2006.
43. Cox S, Mann C, Markham J, Bell H, Gustafson J, Warmington J, et al. The mode of antimicrobial action of the essential oil of Melaleuca alternifolia (tea tree oil). J Appl Microbiol. 2000;88(1):170–5.
44. Liang R, Xu S, Shoemaker CF, Li Y, Zhong F, Huang Q. Physical and antimicrobial properties of peppermint oil nanoemulsions. J Agric Food Chem. 2012;60(30):7548–55. https://doi.org/10.1021/jf301129k.
45. Severino R, Ferrari G, Vu KD, Donsì F, Salmieri S, Lacroix M. Antimicrobial effects of modified chitosan based coating containing nanoemulsion of essential oils, modified atmosphere packaging and gamma irradiation against Escherichia coli O157:H7 and Salmonella typhimurium on green beans. Food Control. 2015;50:215–22. https://doi.org/10.1016/j.foodcont.2014.08.029.
46. Low WL, Kenward K, Britland ST, Amin MC, Martin C. Essential oils and metal ions as alternative antimicrobial agents: a focus on tea tree oil and silver. Int Wound J. 2016;14(2):369–84. https://doi.org/10.1111/iwj.12611.
47. Le Ouay B, Stellacci F. Antibacterial activity of silver nanoparticles: a surface science insight. Nano Today. 2015;10(3):339–54. https://doi.org/10.1016/j.nantod.2015.04.002.
48. Yu BP. Cellular defenses against damage from reactive oxygen species. Physiol Rev. 1994;74(1):139–63.
49. Guzman M, Dille J, Godet S. Synthesis and antibacterial activity of silver nanoparticles against gram-positive and gram-negative bacteria. Nanomedicine. 2012;8(1):37–45. https://doi.org/10.1016/j.nano.2011.05.007.
50. Madigan MT, Martinko JM, Dunlap PV, Clark DP. Brock biology of microorganisms12th edn. Int Microbiol. 2008;11:65–73.
51. Amro NA, Kotra LP, Wadu-Mesthrige K, Bulychev A, Mobashery S, Liu G-y. High-resolution atomic force microscopy studies of the Escherichia coli outer membrane: structural basis for permeability. Langmuir. 2000;16(6):2789–96. https://doi.org/10.1021/la991013x.
52. Brown S, Santa Maria JP Jr, Walker S. Wall teichoic acids of gram-positive bacteria. Annu Rev Microbiol. 2013;67(1):313–36. https://doi.org/10.1146/annurev-micro-092412-155620.